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Review

Molecular mechanisms of aquaporin biogenesis by the endoplasmic reticulum Sec61 translocon

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Abstract

The past decade has witnessed remarkable advances in our understanding of aquaporin (AQP) structure and function. Much, however, remains to be learned regarding how these unique and vitally important molecules are generated in living cells. A major obstacle in this respect is that AQP biogenesis takes place in a highly specialized and relatively inaccessible environment formed by the ribosome, the Sec61 translocon and the ER membrane. This review will contrast the folding pathways of two AQP family members, AQP1 and AQP4, and attempt to explain how six TM helices can be oriented across and integrated into the ER membrane in the context of current (and somewhat conflicting) translocon models. These studies indicate that AQP biogenesis is intimately linked to translocon function and that the ribosome and translocon form a highly dynamic molecular machine that both interprets and is controlled by specific information encoded within the nascent AQP polypeptide. AQP biogenesis thus has wide ranging implications for mechanisms of translocon function and general membrane protein folding pathways.

Keywords: Aquaporin; Biogenesis; Sec61; Endoplasmic reticulum; ER; Translocon; Polytopic protein

Contents

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1. Introduction

Recent advances in aquaporin structure and function have fundamentally changed our views of how water is transported across biological membranes. Cloning and characterization of the first definitive water channel [1,2], CHIP28 (Aquaporin 1), confirmed early studies regarding the proteinaceous nature of the transporter [3–5] and initiated the birth of a rapidly expanding field that has touched broad aspects of biology. It is now clear that water channels are widely expressed throughout prokaryotic and eukaryotic kingdoms, and that they play a major role in normal human physiology and disease [6–8]. Initial insight into the selective basis of water transport provided by Cryo-EM studies [9–11] has been refined by high resolution crystal structures to reveal the mechanism of water and glycerol selectivity at a molecular level [12–15]. With the rapid maturation of this field, new challenges and questions have emerged. For example, much remains to be learned about the precise role of aquaporin expression, regulation, and intracellular trafficking in disease states. Details of AQP structure have also highlighted a particularly perplexing question. Namely, how are functional AQP molecules generated in living cells? This question has specific relevance because inherited mutations in AQP2 cause nephrogenic diabetes insipidus [16,17] by disrupting early biogenesis events and thereby generating unstable structures that are recognized and degraded by cellular quality control machinery [18–20]. Currently, we have only a rudimentary understanding of normal AQP folding pathways and virtually no idea how these pathways are corrupted by disease-related mutations. This review will therefore attempt to summarize our current understanding of AQP biogenesis and provide insight into this particularly challenging aspect of aquaporin biology.

2. Role of the translocon in aquaporin biogenesis

2.1. Aquaporin structure

Aquaporins comprise a ubiquitous family of proteins that contain six transmembrane segments (TMs) arranged in an inverse two-fold pseudo-symmetry around a central water-conducting pore [21,22]. While AQPs generally exhibit a tetrameric quaternary structure [23], each monomer possesses an independent water-conducting channel. Early topologic studies demonstrated that AQP N- and C-termini reside in the cytosol [24–26], and TMs are connected by two relatively short intracellular loops (ICL1, 2) and three extracellular loops (ECL1, 2, 3, Fig. 1A). ICL1 and ECL3 each contains a canonical NPA motif (β-turn) and a half-helix that partially cross the membrane and provide key residues for water and glycerol selectivity [12,13,21]. Thus, the majority of mammalian aquaporin protein is deeply imbedded within the plane of the lipid bilayer, while the remainder is located in either cytosolic or extracytosolic environments (Fig. 1B). The hydrophobic nature of AQPs and their elaborate transmembrane architecture therefore requires a precise mechanism for localizing peptide regions into multiple cellular compartments as well as efficient folding and packing of TMs within the membrane.

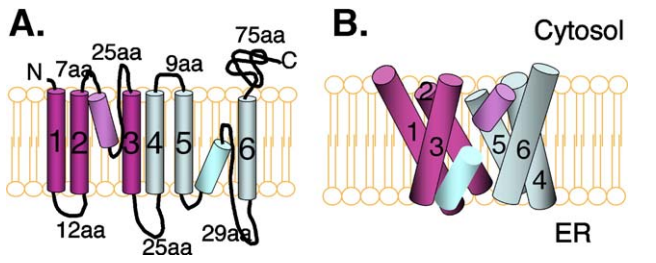

Fig. 1. Aquaporin structure. (A) Transmembrane topology of AQP1 showing relative orientation of N- and C-termini, TM segments, and half helices. Two "half helices" dip partially into the membrane, and their N-termini comprise the canonical NPA motifs necessary for water permeation. Both N- and C-termini of native AQPs reside in the cytosol. (B) 3D arrangement of TMs based on crystal structure of Sui et al. [13]. Note that N- and C-terminal half helices are partially aligned along their major axis and together form an integral part of the outer ring of the water-conducting pore.

2.2. General models of translocon structure and function

Like most eukaryotic polytopic proteins, AQP biogenesis is facilitated by highly specialized folding machinery in the endoplasmic reticulum (ER) [27,28]. A central component of this machinery is the Sec61 translocon [29,30], a large ovoid disc ~100 Å in diameter [31,32] that spans the ER membrane and serves the principal function of translocating nascent polypeptide into the ER lumen and integrating TMs into the lipid bilayer. The translocation channel itself is comprised at least in part by the heterotrimeric protein Sec61αβγ [33–35]. Fully assembled translocons contain multiple copies of Sec61 [36], as well as numerous other associated proteins that include signal peptidase complex (SPC), oligosaccharyltransferase (OST), *TR*anslocation *A*ssociated *M*embrane protein (TRAM) and *TR*anslocation *A*ssociated *P*rotein (TRAP) [27,37–40]. Ribosomes bearing secretory and membrane proteins are usually targeted to the ER very early during synthesis as signal recognition particle (SRP) binds a signal sequence at the N-terminus of the nascent peptide, docks at its ER receptor, and transfers the entire ribosome nascent chain complex (RNC) to Sec61α [30,41]. In the case of mammalian AQPs, the first TM facilitates membrane targeting upon emerging from the ribosome [26,42] when less than 25% of the protein has been synthesized. Thus, AQP translocation and membrane integration are temporally coupled to synthesis of the nascent chain by the ribosome–translocon complex (RTC). A major challenge currently facing biologists is to understand how these events are orchestrated within the context of this large and complex molecular machine (Fig. 2).

Cryo-EM studies of detergent solubilized RTCs have indicated that the central axis of the translocon is directly aligned with the exit tunnel of the 60S ribosomal subunit [43–45]. This is consistent with crosslinking studies demonstrating that the nascent polypeptide encounters ER translocation machinery (Sec61, TRAM) as soon as it emerges from the ribosome [46–50]. As the signal sequence contacts Sec61, it initiates translocation by opening a channel within the

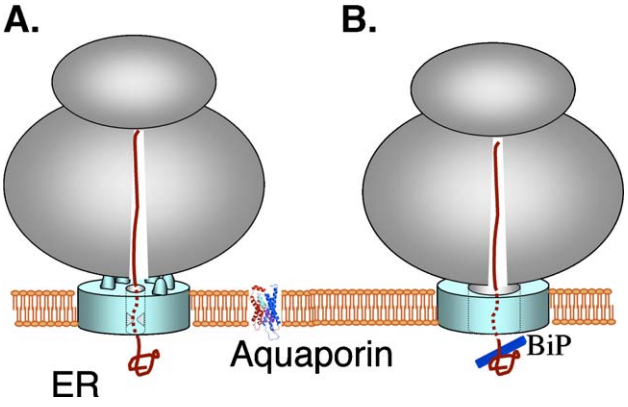

Fig. 2. General architecture of the ribosome–translocon complex. (A) Structural model based on cryo-EM studies of detergent solubilized complexes showing ribosome (gray) bound to the translocon (blue) at numerous points of contact (translocon projections). A gap of ~12 Å is apparent between the complexes. The ~10 Å putative translocon pore (internal dotted cylinder) is proposed to be gated by a helical plug (not shown) and a central ring of hydrophobic residues (hydrophobic gasket, gray triangles). (B) Alternate model of the RTC derived from fluorescence quenching experiments performed on fully assembled and functionally engaged RTC in native ER membranes. The ribosome is shown docked onto a translocon containing a large central pore that is permeable to iodide ions and other aqueous agents. The ribosome exit tunnel and translocon pore form a continuous aqueous pathway that is inaccessible to the cytosol. This seal is proposed to be maintained by tight binding of the ribosome to the translocon [51,52]. During synthesis of cytosolic polypeptide domains, the ribosome–translocon junction is relaxed, and closure of the translocon pore is facilitated by the ER lumenal chaperone BiP (blue bar) which is also required for translocation in yeast [99,100,108,110]. The ribosome and translocon are drawn to approximate relative scale. A folded monomer of AQP1 is also shown in the membrane (panel A) for comparison.

translocon to create a continuous gated aqueous translocation pathway that extends from the ribosome exit tunnel to the ER lumen [51–53]. Experiments with fusion proteins have demonstrated that TM1 from both AQP1 and AQP4 efficiently opens this channel to initiate translocation of the first extracellular loop [26,42]. While it is generally agreed that the translocation pathway is lined at least in part by Sec61α and that Sec61α is one of the first proteins encountered by the newly synthesized nascent chain, the actual composition and dimensions of the translocation channel remain controversial. Crystal structures of a purified, solubilized Sec61 homolog (SecYEβ) derived from *Methanococcus* have suggested that translocation takes place through a relatively small pore (8–12 Å) formed by a single Sec61 heterotrimer (Fig. 2A) [33]. In contrast, fluorescence quenching experiments using assembled and functional translocons in native ER membranes have indicated that the nascent polypeptide is located within a much larger pore (~40 Å) (Fig. 2B) [53]. Further work is therefore required to define the precise composition and physical environment of the translocation pathway in functional and intact translocons.

3. Mechanistic aspects of AQP topogenesis

There are two central questions regarding early aspects of aquaporin biogenesis. How is AQP topology established across the ER membrane? And how are AQP TMs inserted, integrated, and subsequently folded within the hydrophobic environment of the lipid bilayer? Several studies have begun to provide a mechanistic basis with which to view this process. Because AQP polypeptide encounters the translocon as it exits from the ribosome, the translocon must actively direct lumenal and cytoplasmic loops into their respective cellular compartments while at the same time ensuring that TMs are correctly inserted into the lipid bilayer. Moreover, this process occurs rapidly as the nascent chain is expelled from the ribosome exit tunnel at a rate of approximately 5-amino-acid residues per second [54,55]. In order for integration to occur in a cotranslational manner (i.e., during synthesis), the translocation pathway must be highly dynamic, tightly controlled, and precisely coordinated with the synthesis of TM segments and peptide loops. Current evidence indicates that this process is orchestrated via reciprocal interactions whereby access of the nascent polypeptide to the ER lumen, cytosol and lipid bilayer is regulated by the RTC [27,56]. In turn, the pathway through the RTC is controlled by specific topogenic information encoded within the nascent polypeptide [57,58]. For example, topology of extracellular loops is established as they translocate into the ER lumen through the open gate of the translocon pore. However, after synthesis of an ECL, translocation must be terminated in order to direct the next peptide loop (ICL) into the cytosol. Similarly, after synthesis of an ICL, the translocation pathway must be re-opened to allow peptide movement into the ER lumen. Thus, one would predict that during the cotranslational assembly of polytopic proteins, specific signals within the nascent protein should open, close, and re-open the translocation pathway and thereby provide selective access of intracellular and extracellular loops to the cytosol and ER lumen, respectively [27].

3.1. AQP4 topogenesis

Studies attempting to address how lumenal and cytosolic access is controlled during AQP biogenesis have examined the ability of TMs to change the direction of translocation as the nascent protein is synthesized within the RTC [26,42,59–61]. In the case of AQP4, TMs 1, 3, and 5 function as signal (anchor) sequences to efficiently open the translocation pathway and direct movement of extracellular loops 1, 2, and 3 into the ER lumen [42,59]. TMs 2, 4, and 6 alternately terminate translocation, close the translocation pathway, and orient intracellular loops 1, 2 and the C-terminus into the cytosol [42]. Thus, as AQP4 TMs are synthesized, the translocation apparatus (RTC) is regulated such that at any given point of synthesis, the nascent polypeptide has only one pathway to follow, either into the ER lumen or into the cytosol (Fig. 3A). In this manner, the six-spanning topology is established efficiently and cotranslationally as the polypeptide emerges from the ribosome. Surprisingly, not all AQPs achieve their topology via this mechanism.

3.2. AQP1 topogenesis

A detailed analysis of AQP1 revealed that some TMs were much “less efficient” at controlling the translocation pathway

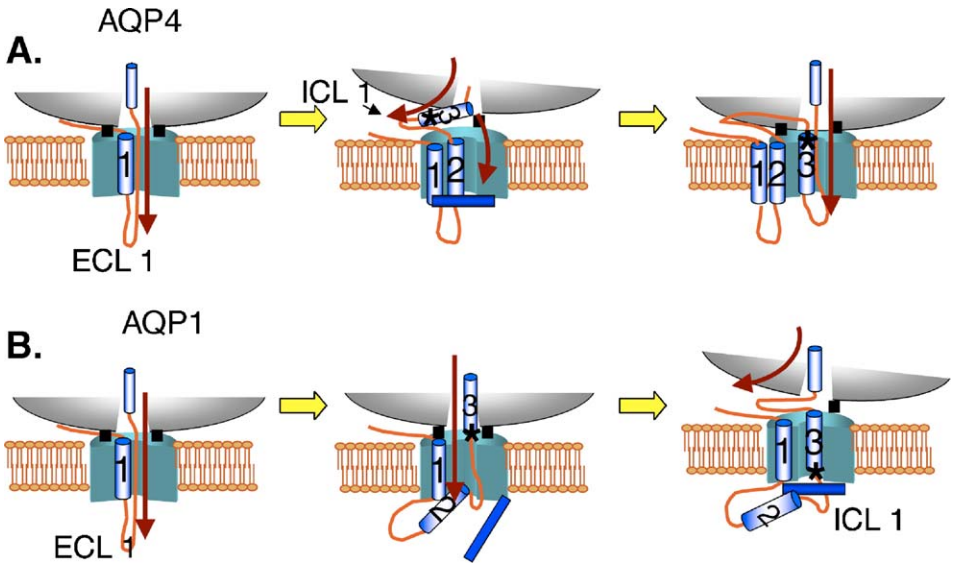

Fig. 3. Different mechanisms of AQP1 and AQP4 topogenesis. (A) AQP4 topogenesis begins as TM1 (blue cylinder) opens the translocon (teal) at the base of the ribosome (gray disc) and initiates translocation of ECL1 through the translocon pore (left panel). Direction of polypeptide movement is shown by maroon arrow. As TM2 enters the translocon (middle panel), it terminates translocation and presumably closes the translocon gate via BiP (dark blue bar). Polypeptide movement is then redirected beneath the base of the ribosome and into the cytosol to establish topology of ICL 1. TM3 exits the ribosome N-terminus first and resets the RTC by opening the translocation pathway into the lumen thus preventing movement of ECL 2 into the cytosol. During this process, TM3 must rotate 180° such that its N-terminus (designated by *) remains facing the base of the ribosome and its C-terminus flanking residues are translocated. In this manner, AQP4 TMs are cotranslationally oriented via the alternating movement of peptide loops into the ER lumen and the cytosol [42]. (B) During AQP1 biogenesis, translocation is also initiated by TM1. However, TM 2 is unable to terminate translocation and transiently passes through the translocon pore together with ICL1. TM3 enters the translocon, terminates translocation, and misdirects ECL2 beneath the ribosome into the cytosol. These events result in TM3 inserting N-terminus first into the translocon and adopting an initial type I topology. Gating of the translocon pore (by BiP) and ribosome–translocon junction are depicted schematically, although the actual mechanisms remain poorly understood.

than their AQP4 counterparts [26]. Specifically, AQP1 TM2 does not efficiently terminate translocation either in its native context or in heterologous chimeric proteins [26,59,60]. As a result, TM2 passes through the translocon and ICL1 transiently enters the ER lumen in >50% of nascent polypeptides. Because TM2 does not close the translocation pathway, TM3 enters an open translocon. Rather than initiating translocation of ECL2, TM3 terminates translocation and initially adopts a type I topology whereby the extracellular loop 2 is mislocalized to the cytosolic face of the ER membrane [26] (Fig. 3B). This unorthodox behavior results in a mixture of nascent chain topologies in which most of the newly synthesized AQP1 molecules initially adopt a four-spanning topology, while only a minority of chains is cotranslationally directed into the six-spanning orientation [26,60]. When first reported, these results caused significant confusion and consternation, as it was difficult to reconcile the initial cotranslationally established AQP1 topology in the ER membrane with the mature, six-spanning topology observed at the plasma membrane [25]. However, subsequent studies using C-terminal translocation reporters as well as inserted epitope tags have demonstrated that the four-spanning topology is actually a folding intermediate that subsequently matures into the six-spanning structure [62]. This is accomplished by an internal 180° rotation of TM3 that converts TM3 from a type I (N_{lum}/C_{cyto}) to a type II (N_{cyto}/C_{lum}) topology and brings TM2 and TM4 into the plane of the membrane (Fig. 4).

Because of the vectoral nature of translation, the N-termini of internal signal anchor sequences (TM3 and TM5) in AQP1 and AQP4 first contact the cytosolic face of the translocon as they emerge from the ribosome (Fig. 3). In the case of AQP4, TM3 cotranslationally acquires its type II (N_{cyto}/C_{lum}) topology such that its N-terminus remains on the cytosolic face of the membrane and its C-terminal flanking residues are directed into the ER lumen (Fig. 3A). Thus, AQP4-TM3 also undergoes a 180° rotation (from downward to upward pointing, see Fig. 3A) relative to the direction of polypeptide movement and its alignment within the ribosome/translocation pathway. A key difference between AQP1 and AQP4

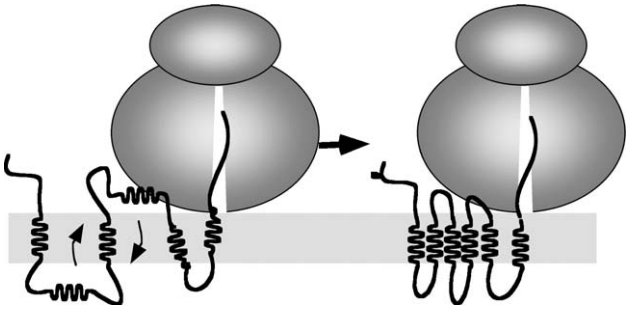

Fig. 4. Mechanism of AQP1 topological maturation. To acquire its mature six-spanning topology, TMs 2, 3, and 4 must be reoriented during and/or following later stages of synthesis. This involves a late 180° rotation of TM3 that converts it from a type I (N_{lum}/C_{cyto}) to a type II (N_{cyto}/C_{lum}) topology and simultaneously positions TM2 and TM4 across plane of the membrane. The efficiency of TM3 rotation is increased as TMs 4, 5, and 6 are synthesized. Thus C-terminal folding information is required for reorienting N-terminal segments. It is currently unknown whether this unexpected folding step takes place within or adjacent to the RTC or after complete integration of the polypeptide into the lipid bilayer.

biogenesis is therefore the timing and synthetic requirements of this inversion event. AQP4-TM3 functions efficiently as an independent type II signal anchor sequence such that orientation occurs cotranslationally and does not require synthesis of downstream TM segments [42,59,63]. In contrast, rotation of AQP1-TM3 occurs at a much later stage of biogenesis and is highly dependent on the synthesis of TMs 4–6 [60,62]. For most signal anchor sequences, TM orientation is rapidly established within the translocon [64] based on the distribution of flanking charged residues (positive-in-rule [65–67]), TM length and hydrophobicity [68], and the folding rate of flanking domains [69]. Analysis of AQP1 and AQP4 chimeras, however, has indicated that primary differences in TM3 translocation activity are caused by variations in C-terminal flanking residues (ECL2) that do not significantly affect these established parameters [59]. Instead, the data indicate that residues within the C-terminal half of AQP1 are primarily needed to properly orient N-terminal TM segments [62,63].

Recent experiments have verified that initial AQP translocation events are similar in cell-free, *Xenopus* oocyte and mammalian cell expression systems, indicating that the unexpected AQP1 folding pathway is widely conserved [60]. There are, however, two caveats to these findings. AQP1 topological maturation in vitro (i.e., conversion from a four- to a six-spanning topology) is dependent upon the source of ER. AQP1 remains in its immature four-spanning topology when translated in traditional canine rough ER microsomes but acquires its mature topology when incorporated into *Xenopus* oocyte-derived ER membranes [62]. At present, the specific ER factors required for AQP1 maturation remain unknown. Second, truncated AQP1 constructs (lacking TMs 4–6) become trapped in the immature topology and are relatively unstable. Thus, immature topological isoforms generated from truncated proteins can only be observed in mammalian cells when examined at very short time intervals after synthesis [60,61].

4. General implications of AQP topogenesis

Studies of AQP topogenesis have several significant implications. First, they demonstrate that translocon gating is not necessarily absolute. Certain TMs that lack strong topogenic properties can direct the translocon into alternate conformations whereby the nascent chain can gain access to either (or both) the cytosolic and lumenal compartments as it exits the ribosome. This contrasts with translocation of most secretory and simple membrane proteins in which signal sequences efficiently direct a uniform topology by establishing a continuous cytosolically inaccessible translocation pathway that extends from the ribosome exit tunnel through the translocon pore [51,52]. It is currently unknown whether a given translocon can provide access to both compartments simultaneously, or whether access is provided in a stochastic manner by adoption of alternate conformations. We favor the latter explanation at this juncture because a translocon open to both ER and cytosol could result in significant mixing of lumenal and cytosolic contents. However, further work is needed to resolve this question.

Second, AQP1 biogenesis has revealed that a mechanism must exist for reorienting TMs and peptide loops that are initially directed into the wrong compartment (Fig. 4). Such a mechanism must provide sufficient flexibility during early stages of biogenesis to allow for "topological editing" while downstream TMs are still being synthesized. Although the mechanism that drives TM3 reorientation remains unknown, it is interesting that this phenomenon is not restricted to AQP1 but has also been observed for other native and engineered eukaryotic polytopic proteins [70–72]. Particularly intriguing, in this respect, are findings that two bacterial transport proteins, lactose permease and phenylalanine permease, can exhibit different topologies depending upon membrane phospholipid composition [73,74]. Both proteins require phosphatidylethanolamine (PE) for function and undergo a reversible topological reorientation of several TM segments and connecting loops when PE is supplied after synthesis has been completed. Thus, the unexpected folding pathway observed for AQP1 may be a relatively common feature of diverse membrane proteins that could be influenced by both the protein machinery and lipid composition of the cell.

Third, a detailed analysis of AQP1 and AQP4 chimeras has demonstrated that the topogenic properties can be dramatically altered by relatively small changes in primary sequence. For example, two residues at the N-terminus of TM2 (N49 and K51 in AQP1 versus M48 and L50 in AQP4) are responsible for the different topological behaviors and cotranslational topologies observed for TM2. Interestingly, N49 and K51 also play a critical role in generating functional AQP1 water channels [59]. Understanding the role of these residues in AQP function may explain why two highly homologous proteins utilize such different folding pathways.

5. Molecular mechanism of membrane integration

Another fundamental requirement for AQP biogenesis is that each TM segment must be integrated into the lipid bilayer. Because polypeptide translocation normally occurs through an aqueous pore, a natural question is whether TM segments actually translocate into the pore and if so, how and when are they transferred into lipid. This point has major implications since early interactions between nascent TM helices (i.e., packing and tertiary structure formation) are profoundly impacted by both general properties of the lipid bilayer as well as interactions with specific lipids [15,75–77].

5.1. Competing models of lateral translocon gating

Two current and somewhat competing models provide a mechanistic explanation as to how integration of a simple, single TM might occur. Both stipulate that in addition to controlling access into aqueous compartments (e.g., lumen and cytosol), the translocon also controls lateral access of the polypeptide into the bilayer. One model proposes that TM segments passively partition into the bilayer based on their affinity for the hydrophobic lipid environment [78] (Fig. 5A). This is consistent with the recent crystal structure of SecYEβ

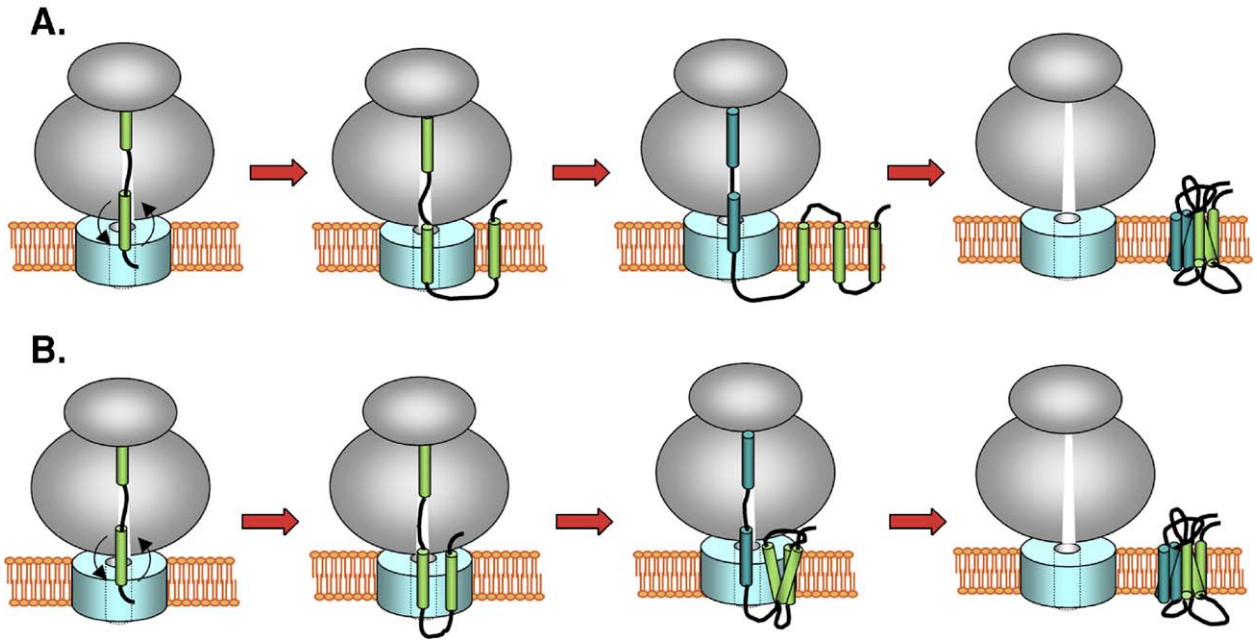

Fig. 5. Alternate models of TM segment integration. (A) In the sequential integration model, TMs (colored cylinders) enter the translocon (blue disc) and rapidly and independently pass through a lateral gate where they equilibrate with the lipid bilayer. Packing of helices and tertiary structure formation would thus take place entirely within the ER membrane. (B) An alternate model is based on evidence that TMs reside within the translocon for prolonged periods of time prior to integration. This model has significant implications for polytopic proteins because early TM interactions and folding events could be impacted by the physical properties of translocon proteins and associated lipids. This latter model predicts that the translocon may provide a unique and more permissive folding environment that enables nascent TMs to sample alternate conformations prior to adapting their final transmembrane structure.

which revealed that the putative 8–12 Å translocation pore can potentially open laterally between the second and seventh TMs of Sec61α by a rearrangement of helices [33,79]. Hydrophobic TM segments could therefore passively move through this lateral opening into the bilayer based on favorable thermodynamic interactions with membrane lipids [77,78]. An alternate model proposes that TM segment integration occurs in a stepwise fashion that is mechanistically controlled and coordinated by the RTC [27,80,81]. In this latter model, TMs pass through and reside within distinct molecular environments within the fully assembled translocon for extended periods of time (Fig. 5B). Release into the bilayer is triggered at specific stages of synthesis and/or at the termination of translation, presumably by conformational changes within the RTC that push out the previous TM [27]. While both models agree that TM segments enter the axial translocon pore and pass laterally into the membrane, the details and mechanism of this lateral movement remain to be reconciled.

6. Integration intermediates define the nascent chain environment

Much current knowledge regarding membrane integration has been derived from biochemical studies of programmed translocation intermediates. When the ribosome reaches the end of a truncated mRNA that lacks an endogenous stop codon, translation ceases, but the polypeptide remains covalently attached to tRNA within the 80S ribosome. Thus, by translating mRNAs truncated at different regions in the coding sequence, it is possible to create synchronized and stable cohorts of nascent chains that reflect the spatial relationships with respect to the RTC at precise stages of synthesis [27]. Early studies examining nascent chain–lipid interactions using alkaline, high salt and urea extraction confirmed that initial stages of translocation were protein mediated and could be temporally dissociated from membrane integration [26,82–86]. In other words, some TM segments including those in AQP1 can achieve a transmembrane orientation without fully integrating into the bilayer. These findings raise questions as to whether TMs might remain within the translocon prior to integration and if so, where this might occur.

6.1. Crosslinking approaches

Bifunctional chemical crosslinking agents and incorporated photoactive probes have begun to provide a more precise view of the timing and mechanism of integration by identifying proteins (and lipids) in the immediate vicinity of the nascent polypeptide. Bifunctional agents typically form covalent bonds between lysine or cysteine residues on adjacent proteins and exhibit relatively high crosslinking efficiencies. However, because they require the close proximity of specific reactive side chains, they do not necessarily identify nearest neighbors if the reactive group is beyond the reach of the spacer arm. They also often generate unwanted secondary and tertiary cross-links between multiple proteins in large complexes. Alternatively, photoactive probes such as 5-azido-2-nitrobenzoyl-lys (ANB-lys) and trifluoromethyl-diazirino-benzoyl-phe (TDB-phe) are introduced during translation at a unique codon that is

recognized by a modified aminoacyl-tRNA [46,49,87,88]. After translation, UV irradiation generates highly reactive radicals that form nonspecific covalent bonds to neighboring molecules. Because nascent chains contain a single photoactive probe, only one crosslink can be formed per polypeptide, and the efficiency of crosslinking directly reflects the proximity of the probe to the target protein. By varying the site of probe incorporation and mRNA truncation, it is thus possible to assess the immediate environment of the polypeptide at virtually any location within the translocation pathway of a fully assembled and functional RTC. This technique provides a non-biased sampling of the nascent polypeptide environment with sufficient resolution to identify components adjacent to different regions (e.g., N- versus C-terminus) as well as different faces of TM helices. Because the reactive radical species have very short half-lives and are prone to solvent quenching, photo-crosslinking yields are generally lower than with bifunctional reagents.

Photo-crosslinking studies have confirmed that secretory proteins contact Sec61α as they pass through the pore, and that crosslinking to Sec61α is lost upon entry into the ER lumen [49,50]. Crosslinking to TMs in bitopic proteins followed several different patterns [47,78,80]. Some TM segments containing TDB-phe were found to crosslink phospholipids almost immediately after contacting Sec61, and lipid crosslinking was stimulated by increasing TM segment hydrophobicity [78,88]. This led to the proposal the TM rapidly passes through the lateral gate into the bilayer. In contrast, other studies have revealed that TMs can remain adjacent to Sec61α and other translocon components during the synthesis of relatively large cytosolic domains (Fig. 5B) [80,81]. These persistent TM interactions exhibit distinct asymmetry wherein residues on different faces of the helix reside in a stable and fixed orientation with respect to specific translocon components. It is difficult to generalize from these studies because few TMs have been examined in detail and because TDB and ANB may have different propensity for lipid crosslinking [81]. However, it would appear from the data that not all TMs proceed directly into the lipid bilayer by a simple partitioning mechanism, and that the translocon contain specific binding sites [89,90] that may transiently accommodate TMs during relatively prolonged periods of polypeptide synthesis.

7. Integration of polytopic proteins

To date few studies have examined how the translocon synchronizes the sequential integration of multiple TMs as they rapidly emerge from the ribosome during polytopic protein synthesis [91–94]. This is particularly important for native proteins such as aquaporins because TMs must not only integrate into the membrane but must also acquire tertiary structure within the lipid bilayer. The early environment experienced by TMs will therefore play a major role in determining how and when helices begin to associate [77]. If TMs disengage from the translocon immediately as they are synthesized, then helical packing would be driven primarily by the physical environment of the lipid bilayer as has been proposed by the two-step model of Popot and Engelman [95]. If, on the other hand, TMs exhibit prolonged interactions with translocon components, then the proteinaceous environment imposed by the translocon could significantly influence the rate and sequence of helical packing and hence the overall folding pathway. Thus, understanding how the translocon controls the early environment of TMs is of more than academic interest and has major implications for diseases in which folding is perturbed by inherited mutations.

7.1. Photo-crosslinking to AQP4 integration intermediates

To investigate this question, we recently performed a systematic analysis of interactions between all six AQP4 TMs and Sec61α during the entire process of synthesis and integration into the ER membrane [94]. The molecular environment of the nascent polypeptide was assessed by examining a comprehensive series of sequentially truncated AQP4 integration intermediates each of which contained a single photoactive crosslinking probe (ANB-lys) at one of three adjacent residues near the center of each TM. The position of probes within the translocation pathway (i.e., distance from the ribosome peptidyltransferase center) was controlled by varying the site of mRNA truncation, and three consecutive probe sites per TM were evaluated to determine the proximity of Sec61α to different faces of the helix. A key element of this analysis was that each truncation site represents a single point of synthesis and thus defines the spatial organization of the nascent polypeptide within the RTC at a single point in time. Crosslinking profiles of Sec61α photoadducts at 18 probe incorporation sites in a total of 204 synchronized integration intermediates thus enabled us to reconstruct dynamic changes experienced by AQP4 TM helices and to develop the first comprehensive description of how TMs enter, traverse, and exit the translocon during synthesis of an entire native polytopic protein (Fig. 6).

Several key findings emerge from this study that bear on both AQP integration and the general role of the translocon in the folding process. First, crosslinking patterns to Sec61α revealed a remarkable coordination of TM entry into, progression through, and exit from the translocon. Each TM moved through the translocon in a unique and highly ordered manner, exhibiting distinct transitions in its relationship to Sec61α that were tightly coupled to the stage of synthesis. As TMs contacted the translocon, they quickly acquired a fixed orientation relative to Sec61α and remained in this “binding” site only until the oriented entry of the next TM. This suggests that the translocon utilizes a specific primary entry site within Sec61α, and that exit from this site is mechanistically coupled to entry of the next TM. Surprisingly, progression of TMs through the translocon was remarkably variable. Some TMs, such as TM2 and TM4, exhibited a single, well-defined period of crosslinking and then abruptly left the proximity of Sec61α. TM2 exhibited robust Sec61α crosslinking during synthesis of only 30 residues while the nascent chain size increased from 110 to 140 residues. Other TMs (e.g., TM1 and TM3) exhibited several distinct phases of crosslinking in which the helix moved sequentially into different molecular environments as

demonstrated by changes in the relative efficiency of crosslinking to different residues around the helix. The most remarkable pattern was observed for TM1 which crosslinked to Sec61α at chain lengths of 80–100, 110–140 and again at a length of 210 residues (Fig. 6).

7.2. Implication for translocon structure and function

During AQP4 biogenesis, multiple TMs were found to reside within and/or adjacent to the translocon for relatively prolonged periods of time. Indeed, at chain lengths of 140 and 210 residues, crosslinking was simultaneously observed for TMs 1–3, and TMs 1, 3, 4, and 5, respectively. While we do not yet know the precise location of each TM relative to all translocon components, these findings raise important questions regarding translocon architecture and function during AQP biogenesis. If translocation takes place through the small pore formed by a single Sec61 heterotrimer, then secondary sites of contact must be located outside the pore. Moreover, if Sec61 molecules are arranged in a back-to-back configuration as has been proposed [79,96], then TMs would exit laterally away from the translocon center and towards the lipid bilayer (Fig. 7A). Given the short length of AQP4 connecting loops and the proposed ~80 Å distance between lateral Sec61 exit sites, this configuration would also require that only one Sec61 complex could be used for the entire synthesis and integration process [79]. Although lipid crosslinking was not detected in our study for technical reasons, this configuration would further suggest that secondary sites of AQP4 interaction take place at the translocon periphery. Alternatively, a recent structural model of two *E. coli* SecYEG complexes docked onto a translating ribosome has suggested a front-to-front arrangement [97]. Although an exit pathway was apparent from only one SecYEG pore, this configuration positions exit sites adjacent to one another such that TMs would initially exit from SecYEG into the space between the heterotrimers prior to reaching the lipid bilayer (Fig. 7B). The proximity of exit sites could also potentially allow multiple heterotrimers to be used for translocation if sufficiently aligned with the ribosome exit tunnel.

Of course fully assembled eukaryotic translocons are more complex and have most recently been proposed to contain up to four Sec61 heterotrimers (as well as accessory proteins) arranged in a large ring-shaped structure [32]. Significant evidence has also indicated that fully assembled and functional translocons contain a large aqueous pore which, during polypeptide translocation, is continuous with the ribosome exit tunnel [51,52,98–100] (Fig. 7C). If eukaryotic Sec61αβγ were

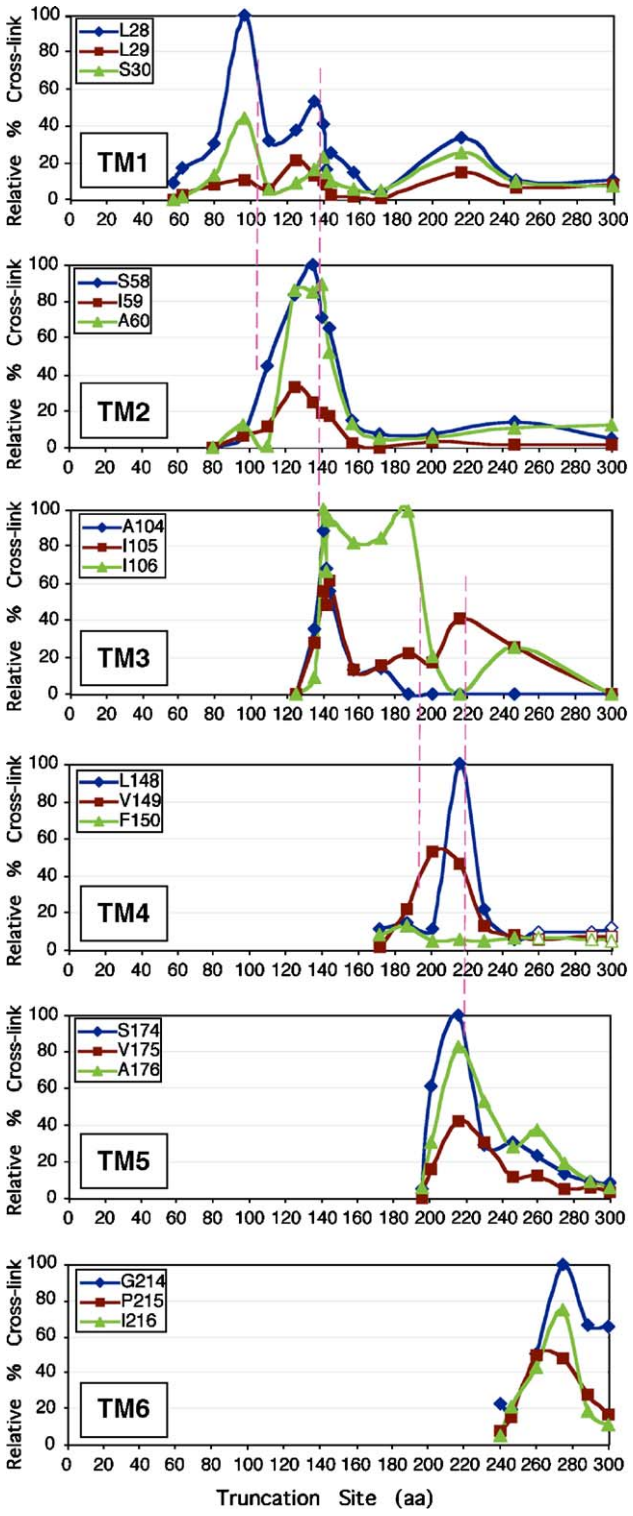

Fig. 6. Sequential triage of AQP4 TMs by Sec61α. Quantitative profile of Sec61α crosslinking to truncated AQP4 integration intermediates. ANB-lys photo-crosslinking probes were incorporated into each AQP4 TM segment at sites indicated (inset) at the upper left of each panel. The *X*-axis of each panel represents the location of mRNA truncation and hence the length of the integration intermediate examined. Stable truncated integration intermediates were synthesized in vitro, and photoadducts to Sec61α were identified and quantitated after crosslinking by immunoprecipitation. In each panel, the amplitude of the curves therefore shows the relative crosslinking intensity (and hence proximity) of residues on different faces of the TM helix at a specific stage of synthesis that corresponds to the site of truncation. When viewed in this manner, it is possible to simultaneously compare the crosslinking profiles and hence spatial relationship of all six AQP4 TMs during synthesis of the entire protein. Note that each TM exhibits a unique pattern of Sec61α crosslinking that reflects its particular pathway through the translocon. Dashed vertical lines show specific stages of synthesis that represent key transitions in the environment of TMs and the coordinated timing of TM entry into and exit out of the primary Sec61 binding site. Note also that multiple TMs were observed to simultaneously crosslink Sec61α at the same nascent chain length. Figure was modified from Sadlish et al. (Ref. [94]).

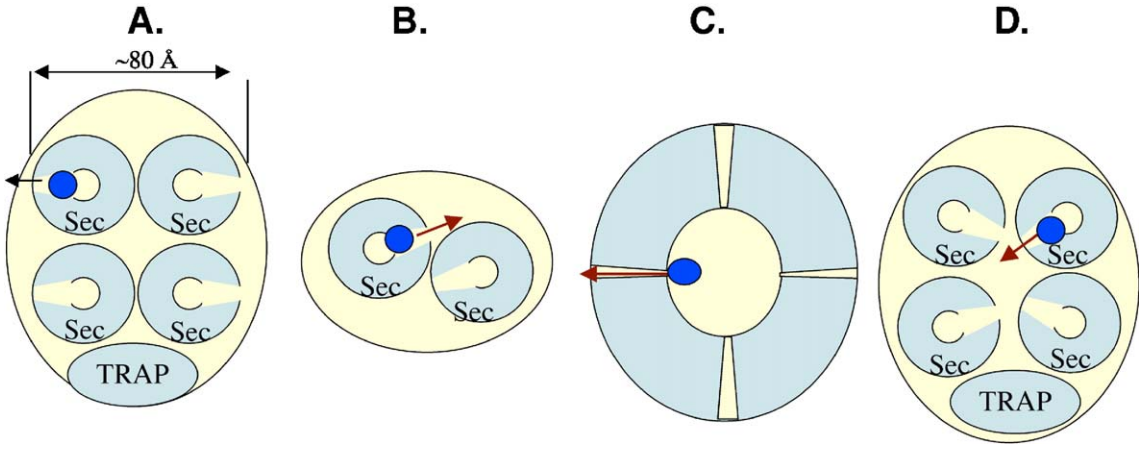

Fig. 7. Models of the Sec61 lateral exit gate. (A) Back-to-back configuration. Possible arrangement of Sec61αβγ heterotrimers (Sec) based on Cryo-EM structure of solubilized ER translocons, 2D crystals of E. coli SecYEG, and high resolution of the *Methanococcus* SecYEβ [32,33,96]. The lateral gate is shown in yellow and is proposed to reside between TM2 and TM7 of SecY. The size of individual heterotrimers would place exit sites ~80 Å from one another [79]. Blue circle represents nascent TM. Approximate size of the translocon (oval) and proposed location of TRAP is indicated. (B) Schematic representation of front-to-front arrangement of SecYEG dimers bound to a translating ribosome [97]. (C) Model of translocon derived from fluorescence quenching experiments showing a large central pore surrounded by oligomeric ring of Sec61 and other translocon proteins [27,51–53]. Lateral exit sites are shown between putative translocon subunits. (D) Hypothetical arrangement of four Sec61 heterotrimers arranged in a front-to-front configuration showing the lateral exit sites of Sec61 heterotrimers oriented towards the center of the complex where helices could potentially reside prior to passage between subunits into the lipid bilayer.

arranged in a front-to-front organization, then Sec61 oligomers could potentially form a ring, and initial exit of AQP4 TM helices from the putative small translocation pore could take place towards the center of the translocon complex (Fig. 7D). Interestingly, this central region was initially visualized as a large open pore [43,45,101], but subsequent higher resolution structures of detergent-solubilized translocons have revealed only a central depression that has been proposed to contain mainly lipid [31,32,44]. Because the functional status and subunit composition of purified translocons are unknown, much remains to be learned regarding the structure of fully assembled eukaryotic translocons. An intriguing possibility, although untested at this point, is that TMs might initially exit from Sec61 into a relatively hydrophobic, lipid-like environment that is physically and chemically distinct from the membrane bilayer and surrounded or partially surrounded by translocon proteins (Fig. 7D).

7.3. Specific challenges for TM orientation and integration

Regardless of the precise arrangement of Sec61 in the assembled eukaryotic translocon, the persistent, selective, and asymmetric binding observed for AQP4 TMs provides strong evidence that helices do not always freely partition and equilibrate individually into the lipid bilayer. Rather, it would appear that some AQP4 helices accumulate at secondary and/or tertiary sites within an environment that is likely comprised of both protein and lipid components. Given that the rate of protein synthesis is remarkably slow when compared to secondary and tertiary structure formation, it is highly likely that early helix-helix interactions take place within this immediate environment. If so, then the translocon could impact early steps of membrane protein folding in unanticipated ways by influencing the composition and/or physical properties (i.e., strain energies) of adjacent lipids [77]. It is tempting to speculate that this might also provide a productive environment for formation and early maturation of folding intermediates such as those observed for AQP1. It will also be quite interesting to determine whether AQP half helices in ICL1 and ECL3 insert directly into membrane lipids or into a protein scaffold formed by other AQP TMs either within the translocon or after full release into the lipid bilayer.

A second feature of AQP folding that must be considered is how TMs are properly oriented within the physical confines of the translocon apparatus. Since the nascent polypeptide exits the ribosome vectorally in an N-to C-terminal direction, TMs 1, 3, and 5 must rotate 180° in order to achieve their correct type II (N_{cyto}/C_{lum}) topology. For AQP4, this rotation occurs sequentially and does not require cooperative interactions between multiple TMs [42,59]. Several lines of evidence have also suggested that TM helices can form very early within the translocation pathway and even within the ribosome exit tunnel [102–105]. Our findings are consistent with this and raise the question as to when and where helix rotation takes place. Both the ribosome tunnel [106,107] and putative Sec61α translocation pore [32] are clearly too small to accommodate rotation of a 30 Å helix. Interestingly, crosslinking profiles revealed that TM3 initially contacts the translocon in a relatively random orientation and then (~10 residues later) enters into a fixed binding site within Sec61α where it remains during synthesis of nearly 80 additional residues. Thus, TM3 rotation occurs either before entry into Sec61 or remarkably late as it transitions into its site of secondary interaction.

Early rotation could conceivably take place at the base of the ribosome, particularly if the ribosome–membrane junction were relaxed coincident with TM2 terminating translocation and initiating movement of ICL 1 into the cytosol [99]. TM3 rotation could be facilitated by electrostatic interactions between basic residues near its N-terminus and residues within Sec61α [64,67]. Alternatively, rotation could conceivably take place in the context of a larger translocon pore or central

location within the fully assembled translocon (Fig. 7C, D) as has been demonstrated by fluorescence quenching experiments [51–53]. In this case, TM3 would reinitiate translocation upon entry into Sec61α by reestablishing the ribosome–translocon junction and opening the translocation pathway [99,100,108]. A third possibility is that TM3 could enter the translocon in a type I topology as has been suggested for other signal anchor sequences and then rotate after its exit into its secondary site of interaction [64,109].

An additional constraint arises with the sequential arrival of TMs 4 and 5, which are separated by a very short connecting loop (~9 residues). Both helices exhibit peak crosslinking at the same stage of synthesis, i.e., at a nascent chain length of 216 residues. However, they do not insert in a loop-wise fashion because TM5 must rotate 180° about its axis to initiate translocation of ECL3. It is difficult to conceive how a single Sec61 heterotrimer could simultaneously accommodate TM4, TM5, TM5 rotation, and a strand of ECL3 given the small confines of a single hourglass shaped pore. Thus, the observation that TMs 4 and 5 simultaneously crosslink Sec61 supports the presence of a larger structure (possibly a large pore) that can accommodate and provide conformational flexibility to relatively large peptide regions. Important questions therefore remain as to where closely spaced helices reside during the orientation and integration process.

8. Conclusions

Advances in our understanding of translocon structure and function, as well as the biogenesis mechanisms of translocon "substrates", have led to various models that attempt to explain translocation across and integration into the ER membrane. At the same time, studies of secretory, transmembrane, and polytopic protein biogenesis have provided key information that must be incorporated into these models. Studies of AQP biogenesis have revealed novel and unexpected folding pathways that begin to explain how AQP characteristic transmembrane structure is formed. These studies also have general implications for both membrane protein folding and mechanisms of translocon function. For example, the sequential entry and exit of AQP4 TMs into a primary binding site is consistent with a relatively small translocation pore that accommodates one helix at a time. In contrast, simultaneous association of multiple helices, the location and timing of helix rotation, and physical constraints imposed by short connecting loops require that any model of translocon structure and function must accomodate specific biogenesis needs of protein substrates.

It is now clear that the translocon is integrally involved in directing early events of AQP biogenesis. Evidence also suggests that its role is not solely limited to translocation of extracellular domains and orientation and integration of TM helices. Rather, the early stages of secondary and possibly tertiary folding are likely initiated within the immediate environment of the translocon prior to release of the entire polypeptide into the lipid bilayer. A full understanding of how these folding events are orchestrated is currently far from our grasp and will undoubtedly require a more precise knowledge of the structural organization of assembled and functional translocons and their specific interactions with the nascent chain. Solving this complex and perplexing problem will require a variety of perspectives and the concerted efforts of individuals using complementary techniques. Ultimately, both complementary and conflicting results must be developed into a unified model that will describe and enable predictions of membrane protein folding pathways with accuracy similar to or greater than that now available for soluble proteins. This information will then facilitate the formidable task of rational therapeutic intervention in situations where the folding pathway has been corrupted by disease-related mutations.

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